Mutagenicity (AMES test) protocol of assessment of Genotoxicity of a product or substance:

For example: New Atraumatic Restorative Treatment (ART) materials incorporated with *Azadirachta indica* (Neem).

Strains: MTCC Salmonella Typhimurium TA 98 and TA 100

Growth condition: Aerobic

Incubation: 48 hours

Sub-culturing period: 30 days

Temperature: 37^0C

Growth media: 114 (Ampicilline media)

Composition of growth medium:

50 x VB salt	- 20ml
40% glucose	-50ml
Sterile Histidine HCl.H_2O (2g per 400ml of water)	-10ml
Sterile 0.5mM Biotin	- 6ml
Sterile ampicilline solution (8mg per ml 0.02N NaOH)	- 3.15ml
Agar	- 15g
Distilled water	-910 ml

Sub-culture:

1. Add 0.3 to 0.4 ml of 114 liquid medium to make a suspension of the culture.
2. Streak a few drops of the suspension to 114 liquid medium (solidified with agar) in a Petri plates and incubate it for 48 hours.

Standard plate incorporation assay without metabolic activation system

The standard plate incorporation assay consists of exposing the tester strains to the test chemical directly on a minimal glucose agar plate (GM plate) usually in the presence and absence of a metabolic activation system.

1. Steps taken prior to performing the experiment
    - Inoculate salmonella cultures in fresh nutrient broth 3 hours prior to performing the experiment.
    - Label an appropriate number of GM agar plates and sterile test tubes for each test samples.
    - Prepare the dilutions of ART sample, positive mutagens (sodium azide for strain TA 100, 4-Nitro-*o*-phenylenediamine for strain TA 98).
    - Melt top agar supplemented with 0.05mM histidine and biotin maintain at 43 ^{0}C to 48 ^{0}C.

2. To the 13x100mm sterile glass tubes maintained at 43^{o}C, add in the following order with mixing after each addition.
    - 2 ml of molten top agar
    - 0.1 ml of the test chemical dilution
    - 0.1 ml culture of the Salmonella strain

3. The contents of the test tubes are thee mixed and poured onto the surface of GM agar plates.
4. When the top agar has hardened (15 min), the plates are inverted and placed in a 37^0 C incubator for 24 - 48 hours.
5. The colonies are then counted and the results are expressed as the number of revertant colonies per plate.

Preparation of reagents- to check mutagen of ART

Nutrient broth to grow the tester strains:

Distilled water-	100ml
Nutrient north-	0.65 g

Autoclave for 20 min. cool and store in dark at room temp.

Vogel-Bonner medium E (50X) For 1 liter medium

Warm distilled H2O (45ºC)	670 ml
Magnesium sulfate (MgSO4, 7H2O)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K2HPO4)	500 g
Sodium ammonium phosphate (NaHNH4PO4, 4H2O)	175 g

The salts were added in the order indicated in the warm water and each salt was allowed to dissolve completely before adding the next. Volume was adjusted to 1 liter and then autoclaved before use for 20 min.

0.5 mM hitidine/biotin solution: Ingredient Per 250 ml

D-Biotin	30.9 mg
L-Histidine.HCl	24.0 mg
Distilled H_2O	250 ml

The solution was sterilized by passing through 0.22-µm-membrane filter or it can be autoclave for 20 min at 121^0C can be stored in glass bottle at 4^0C.

Top agar supplemented with his/bio. Ingredient Per 1 liter

Agar	6 g
NaCl	6 g
0.5mM hitidine/biotin solution	100 ml

Add Agar and NaCl to water (900ml) autoclave it for 30 min. Cool it to 50^0C then add 100ml of 0.5mM hitidine/biotin solution.

40% Glucose in 100 ml

Dextrose	40 g
Distilled water	100 ml

Take 40 g of dextrose in a 100 ml volumetric flask and add distilled water. Transfer to conical flask and then autoclave it for 20 mins.

Minimal glucose plate Ingredient Per 1000 ml

Agar	15 g
Distilled H2O	930ml
50 X VB salts	20 ml
40 % glucose	50 ml

15 gm of agar was added to 930 ml of distilled H2O and autoclaved for 20 min. when the solution has cooled slightly, 20 ml of sterile VB salts and 50 ml of 40% glucose were added. After addition of all the ingredients the solution should be stirred thoroughly then was poured 30 ml of it into each petri plate. The 50X VB and 40% glucose were autoclaved separately.

References:

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Modified and adjusted based of generic protocol of Ames test for ART material

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